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Prediction of linolenic and linoleic fatty acids content in flax seeds and flax seeds flours through the use of infrared reflectance spectroscopy and multivariate calibration

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ABSTRACT

Infrared spectroscopy was used for multivariate determination of linoleic (LA) and linolenic (ALA) acids in yellow and brown flax seed samples, using partial least square regression (PLSR). The models were developed by correlating near-(NIR) and mid-infrared (MIR) spectroscopic signals with the acid content determined by GC-FID. For the flax seed samples, the best models for both, LA ($R^2 = 0.90$, Standard Error of Prediction (SEP) = 1.61) and ALA ($R^2 = 0.86$, SEP = 0.63) were obtained by processing the NIR spectral data. For samples of flax seed flours, the best models for prediction of the ALA ($R^2 = 0.99$, SEP = 1.21) and LA content ($R^2 = 0.88$, SEP = 0.76) were developed by processing the NIR and MIR spectral region, respectively. This report demonstrates that NIR and MIR spectroscopies are efficient techniques for the determination and quantification of LA and ALA in flax seed and flax seed flours.

1. Introduction

Several anthropological, genetic and nutritional studies have suggested that human beings evolved on a diet containing a ration of ω−6 to ω−3 fatty acids (FA) of about 1/1. However, nowadays Western diets have a ratio of 10/1 to 20–25/1, indicating that Western diets are deficient in ω−3 FA compared to the diet on which humans evolved (Simopoulos, 2011). Several decades ago, Burr and Burr (1998) demonstrated the importance of linoleic acid (LA) 18:2ω−6 and alpha-linolenic acid (ALA) 18:3ω−3 in restoring the effects caused by the fat-free diet in deprived animals. ALA and LA are important components of cell membranes. These two essential FAs are not interconvertible in the human body, are metabolically and functionally distinct, and often present important opposing physiological roles (Simopoulos, 2006). A diet rich in ω−6 FAs shifts the physiological state towards one that is prothrombotic and proaggregatory, with enhancement in blood viscosity, vasospasm, and vasoconstriction and decreases in bleeding time (Simopoulos, 2006). Deficiency of ALA is also known to increase incidence and severity of inflammatory/hyperproliferative diseases (Fan & Chapkin, 1998). Thus, a lower LA-to-ALA ratio dietary intake is necessary for the prevention and amelioration of chronic diseases. Seeds from the flax plant (*Linum usitatissimum* Linaceae) are known to be one of the richest sources of ALA (Prieto et al., 2012). Not surprisingly, flax seed consumption has been considered to help in disease prevention such as arthritis, diabetes and menopausal symptoms, besides reducing the risk of coronary heart diseases, stroke and cancer (Morris, 2007a).

The FA composition of oilseeds is usually determined by gas chromatography (GC). However, this method of analysis is destructive, time-consuming and expensive (Fassio & Cozzolino, 2004). In addition, GC requires a long time for sample preparation, derivatization, and analysis by highly-skilled personnel, besides not allowing analysis on-line (Cantarelli, Funes, Marchevsky, & Camina, 2009; Guy, Prache, Thomas, Bauchart, & Andueza, 2011; Prieto et al., 2012; Sinelli et al., 2010). Because of the demand for easy and fast analytical methods, both near infrared (NIR) and mid-infrared (MIR) spectroscopies have been considered as an interesting alternative to traditional analytical techniques, GC included (Casale, Zunin, & Cosulich, 2010; Zhang, Cheng, Liu, He, & Frost, 2011). NIR and MIR infrared spectroscopies offer a number of important advantages over methods traditionally used for chemical analysis. Infrared spectroscopy is non-destructive, rapid and can analyze large number of samples in small quantities, requiring minimal or no sample preparation. Moreover, infrared spectroscopy is less expensive, because no reagents are required, is environmentally friendly, because no waste is produced (Allendorf, Subramanian, & Rodriguez-Saona, 2011; Casale et al., 2010; Patil, Oak, Taware, & Tamhankar, 2010), and provides more information about the components present in the raw materials and formulated food products (Sundaram, Kandala, Holser, Butts, &

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Windham, 2010). Because of that, infrared spectroscopy has been used as an analytical tool to determine several constituents present in food like carbohydrates, proteins and lipids (Di Egidio et al., 2009; Sundaram et al., 2010).

There are two varieties of flax seeds used regularly in human diet: the yellow and brown flax seeds (Morris, 2007b; Pradhan, Meda, Rout, Naik, & Dalai, 2010). The fact that FA composition in flax seeds changes between varieties and also according to growth environment and seed processing (Daun, Barthet, Chornick, & Duguid, 2003) prompted us to search for a rapid, easy and less expensive analytical method, which also allowed analysis on-line, for the determination of LA and ALA in flax seeds. In this paper, we describe the development multivariate calibration models capable to predict LA and ALA contents, in yellow and brown flax seeds and flax seed flours, through the use of NIR and MIR reflectance spectroscopy.

2. Materials and methods

2.1. Materials

A total of 78 samples of flax seeds and flax seed flours were obtained from two local food-suppliers, Jasmine Limited (Curitiba, PR, Brazil) and Nutri House Foods Limited (Curitiba, PR, Brazil). The samples were divided into two groups: one group contained 12 yellow flax seed flour samples and 10 yellow flax seed samples, both from Jasmine Limited. The second group contained 14 yellow and 14 brown flax seed flour samples, and 14 yellow and 14 brown flax seed samples, all from Nutri House Foods Limited.

2.2. Lipid extraction

Total lipids were extracted by mixing 3 g of each sample with petroleum ether (Carlo Erba, Milan, Italy) for 5 h using a Soxhlet (Prodicil, Curitiba, PR, Brazil) solvent extractor, according to the American Oil Chemists' Society Official Method, Af 3–54 (AOCS, 2004).

2.3. Analysis of fatty acids

Total fatty acids (FA) were extracted and converted into their methyl ester derivatives. Briefly, 10 mg of total lipids from flax seeds and flax seed flours was dissolved in 1 mL of *n*-hexane (Mallinckrodt Chemicals, Saint Louis, MO, U.S.A.). Then, the solution was derivatized with diazomethane for approximately 120 min, according to the manufacturer's recommendation (Sigma-Aldrich Co., Saint Louis, MO, U.S.A.). The composition of the derivatized FAs was determined by gas chromatography–mass spectrometry (GC–MS) and their content was determined by gas chromatography-flame ionization detection (GC-FID), as described below.

2.4. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis was performed using a Varian-450 gas chromatograph combined with a Varian-320 Mass Spectrometer (Agilent Tecnologies, Oxford, U.K.) according to Yang et al. (2010), with modifications. For the gas chromatography, a CP 8944 capillary column (30.0 m × 0.25 mm × 0.25 μm) (J&W Scientific, Agilent Technologies, Santa Clara, CA, U.S.A) was used. All of the parameters used for the GC–MS runs were optimized during this study. Oven temperature program: the column was held initially at 190 °C for 1 min. Then, the temperature was increased to 200 °C, at 10 °C/min and held for 2 min at 200 °C. Afterwards, the temperature was increased to 310 °C at 3.5 °C/min. Then, the temperature was held for 1.57 min at 310 °C. The injector temperature was 315 °C. Helium was used as carrier gas at a flow rate of 0.8 mL/min and split ratio 1:50. Injection volume was 1 μL. Mass spectrometry conditions were: ionization energy at 70 eV, ion source temperature at 330 °C and mass range at 32–380 amu.

FA methyl esters were identified by their characteristic electron impact MS spectra (NIST library, National Institute of Standards and Technology, Gaithersburg, MD, U.S.A.) and retention times (R_t), which were compared with those of methyl esters of primary standards (Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) and Supelco™37 standard FAME Mix (Supelco Inc., Bellefonte, PA, USA).

2.5. Gas chromatography (GC)

GC analysis was performed using a Shimadzu GC 14B gas chromatography equipped with a flame ionization detector (FID) (Shimadzu Co., Kyoto, Japan) and a DB-23 capillary column (60 m × 0.25 mm × 0.25 μm) (J&W Scientific, Agilent Technologies, Santa Clara, CA, U.S.A), according to Petrović, Kezić, and Bolanča (2010), with modifications. All of the parameters used for the GC run, described below, were optimized during this study. The injector and detector temperatures were 235 and 260 °C, respectively. Inlet pressure was 250 kPa, linear gas velocity was 14.5 m/s and split ratio was 1:63. Nitrogen was used as a carrier gas at a flow rate of 0.71 mL/min, with injection volumes of 1 μL and 2 μL, respectively for flax seed flours and flax seeds FA methyl esters. Baseline separation was achieved at an oven temperature of 220 °C and running time of 11 min. FA esters were quantified in GC runs by comparison with the retention times of methyl esters of primary standards (Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) and Supelco™37 standard FAME Mix (Supelco Inc., Bellefonte, PA, USA). Quantification was carried out by normalization and the data obtained were converted to a percentage of the total FAs measured.

2.6. Infrared reflectance spectrometry

The reflectance spectra of yellow and brown flax seeds in the near- (NIR) and mid-infrared (MIR) range were measured on flax seeds and flax seed flours. Four gram samples were scanned, in the reflectance mode, in the 9000–4000 cm^{-1} range for the NIR spectra, while 1 gram samples were scanned, in the 4000–750 cm^{-1}, range for the MID spectra using a Tensor 37 FTIR spectrometer system (Bruker Optics, Ettlingen, Germany) equipped with an integrative sphere. OPUS software (v. 6.0 Bruker Optics, Ettlingen, Germany) was used for spectral acquisition and instrumental control. Reflectance data were recorded, at a nominal resolution of 4 cm^{-1}, accumulating 128 scans, for both NIR and MIR spectra.

2.7. Multivariate calibration

The NIR and MIR spectral data and the FAs content data were organized in matrices using the Origin Pro 8.0 software (OriginLab., Northampton, MA, U.S.A.). Principal Components Analysis (PCA) was carried out through the use of PLSR-Toolbox 3.0 (Eigenvector Research Inc., Manson, WA, U.S.A.) in MATLAB (v 7.0, The MathWorks Inc., Natick, MA, U.S.A.) software, using NIR and MIR spectral data from the whole set of samples, i.e. 38 seed samples and 40 flour samples. For both, the NIR and MIR regions, differences were found for lipid content of flour and seed samples. For the NIR region, the first Principal Component (PC1) explained 75.60% of the variation among samples. For the MIR region, the second principal component (PC2) explained 20.34% of the variation. Because of the above mentioned differences in lipid content found for flour and seed samples, we decided to develop multivariate calibration models specific for flours and seeds.

In order to optimize the calibration accuracy, different combinations of scattering corrections and mathematical pre-treatments were used to reduce systematic noise, such as baseline variation. The most effective pretreatments for the spectral data were Multiplicative Scatter Correction (MSC) plus Normalization, MSC plus Smoothing and Normalization, Standard Normal Variate (SNV) plus Detrend, and MSC plus Smoothing and First Derivate (Savitzky–Golay's method, gap size = 11 data points).

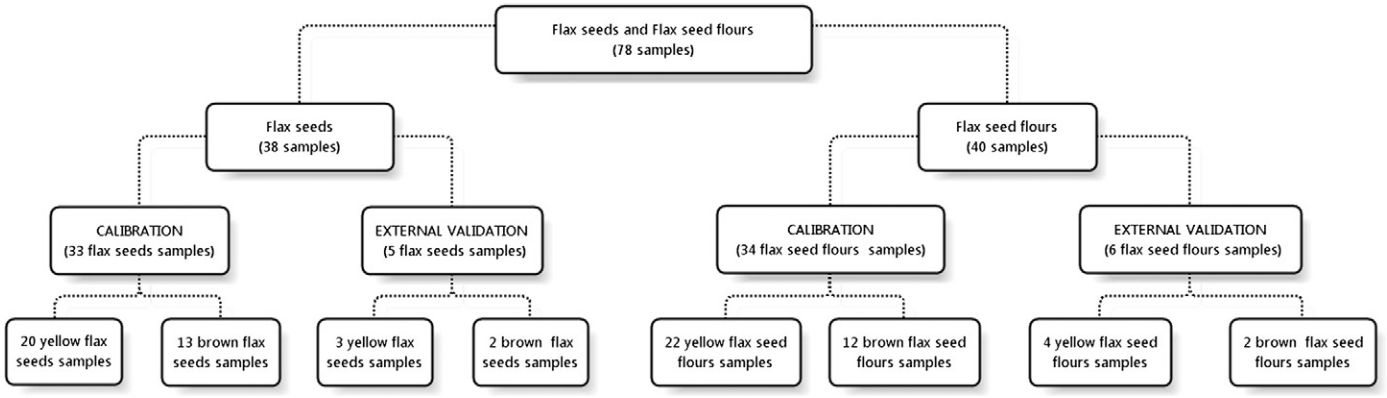

Fig. 1. Summary for the sampling used for calibration and external validation of the fatty acid data.

Sampling used for calibration and external validation is summarized in Fig. 1. The model performance was evaluated through the analysis of the number of Latent Variables (LVs) and Standard Error of Cross-Validation (SECV), and also by Standard Error of Prediction (SEP) and the coefficient of determination for validation (R^2), which describes the relationship between values predicted by the models and values obtained through the use of the reference method (GC-FID).

3. Results and discussion

3.1. Fatty acid composition as measured through GC-FID and GC–MS, the reference methods

Fig. 2 shows the main FAs found in a representative flax seed flour sample. Linoleic (LA, C18:2), linolenic (ALA, C18:3), oleic (C18:1), palmitic (C16:0) and stearic (C18:0) were the major FAs, identified by both, GC–MS and GC-FID. GC–MS and GC-FID chromatograms for a flax seed sample were very similar to the ones presented for the flax seed flour sample (not presented). Quantification of ALA and LA methyl esters in 78 flax seeds samples by GC-FID demonstrated that the ALA content ranged from 42.5% to 55.6% in flax seed flours and from 47.1% to 48.7% in flax seeds, while the LA content ranged from 17.9% to 23.6% in flax seeds flours and from 15.4% to 21.0% in flax seeds.

3.2. Development of multivariate calibration models

Fig. 3 displays the NIR and MIR spectra for flax seeds flours (a) and flax seeds (b) samples. For flax seed flours, thirty-four spectra were used for calibration while six spectra were used for external validation. For flax seeds, thirty-three spectra were used for calibration while five spectra were used for external validation. Several partial least squares regression (PLSR) models were built in order to achieve a high prediction capacity. The use of several latent variables along with various methods of signal pre-processing allowed the development of multivariate models presenting high coefficients of determination for predicted and experimental values.

For flax seed flours, the best multivariate calibration models developed for ALA content used data from the NIR spectral region, while the best model for LA content used data from the MIR spectral region. For flax seeds, the best models for both LA and ALA contents used data from the NIR spectral region (Table 1). For the prediction of the ALA content in flax seed flours, the best developed model used two latent variables which described 100% of the spectral data and 99.98% of the ALA content, with Standard Error of Prediction (SEP) of 1.21%, Standard Error of Cross-Validation (SECV) of 0.64% and coefficient of determination for validation (R^2) of 0.99. Multiplicative Scatter Correction (MSC), which corrects light scattering caused by irregularities in solid samples, and normalization, which equalizes all of the variables in terms of magnitude, were used as pre-treatments for the spectral data. For the prediction of the ALA content in flax seeds, the best developed model used four latent variables which described 99.97% of the spectral data and 100% of the ALA content, with SEP of 0.63%, SECV of 0.26% and R^2 of 0.86. MSC, smoothing,

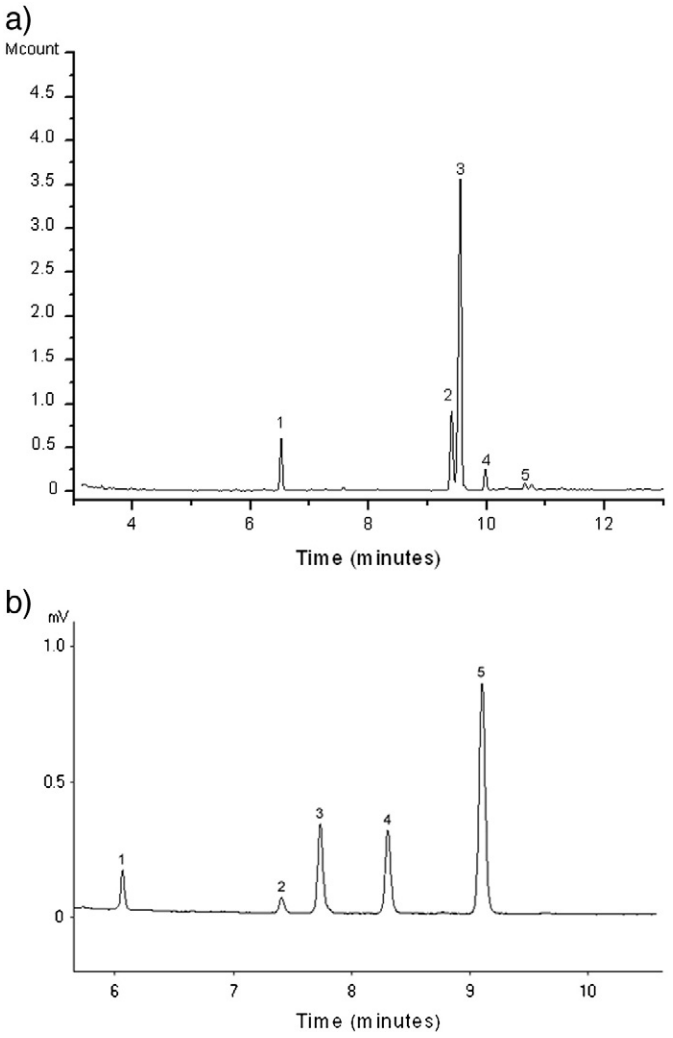

Fig. 2. (a): GC–MS chromatogram of main methyl esters of fatty acids palmitic acid (1), linoleic acid (2), alpha-linolenic acid (3), stearic acid (4) and oleic acid (5) from a representative flax seed flour sample. (b): GC-FID chromatogram of main methyl esters of fatty acids palmitic acid (1), stearic acid (2), oleic acid (3), linoleic acid (4) and alpha-linolenic acid (5) from a representative flax seed flour sample.

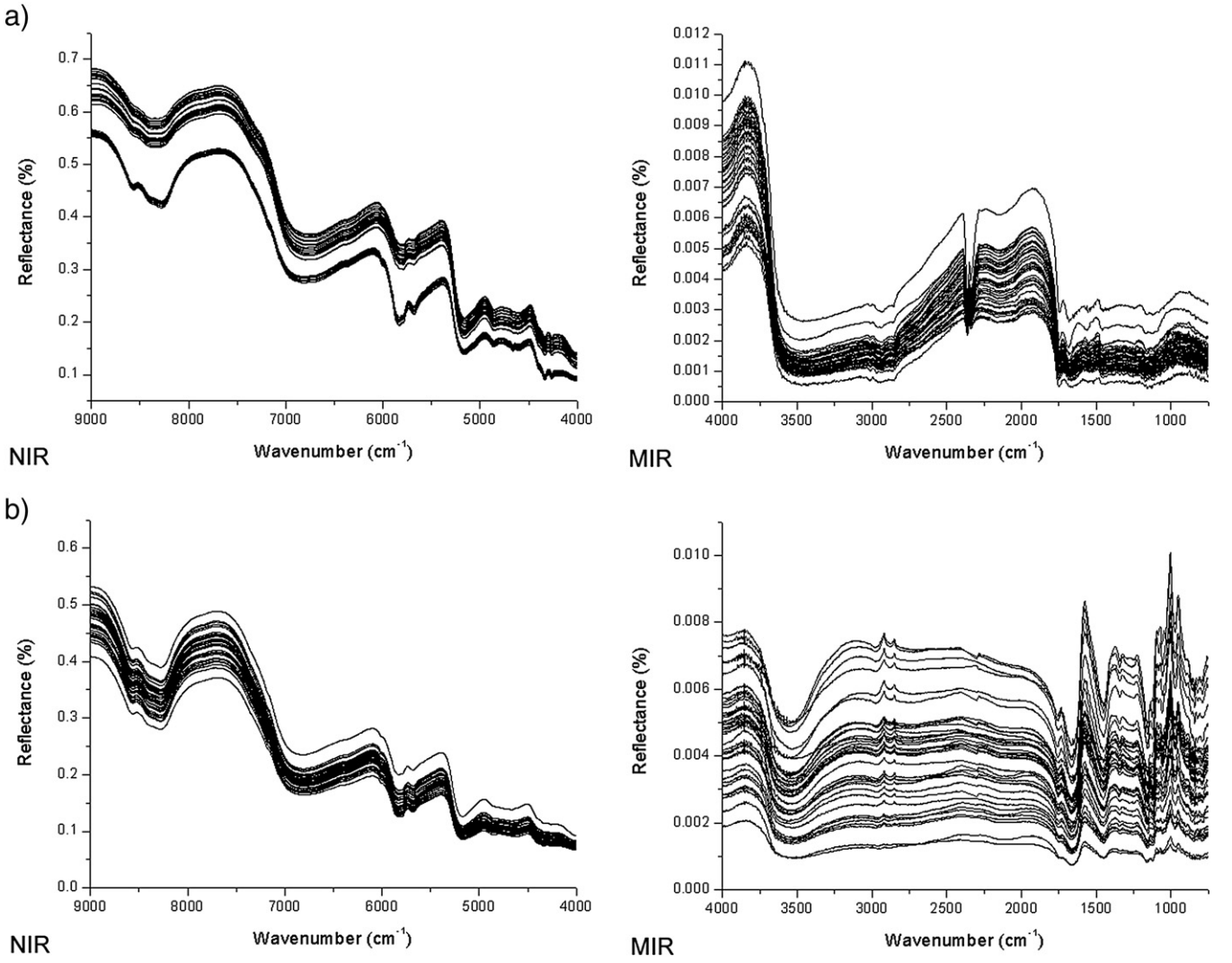

Fig. 3. NIR and MIR raw spectra of the flax seed flour samples (a) and flax seed samples (b), respectively.

which minimizes noise, and first derivative transformation, which highlight relevant analytical signals of the spectral data, were used as pretreatments for the spectral data.

The best model developed for the prediction of the LA content in flax seed flours used three latent variables, which described 99.97% of the spectral data and 99.83% of the LA content, with SEP of 0.76%, SECV of 0.95% and R^2 of 0.88. MSC, smoothing and normalization were used as pretreatments for the spectral data. For the prediction of LA in flax seeds, the best model used three latent variables. MSC, smoothing and the first derivative transformation of the spectral data were used as pretreatments for the spectral data. The calibration model described 99.96% of the spectral data and of 99.85% of the LA content, with SEP of 1.61%, SECV of 0.76% and R^2 of 0.90. Fig. 4 presents a scatter plot showing the relationship between the reference value and predicted value for LA (a) and ALA (b) contents in flax seeds, using NIR and MIR spectroscopies, respectively. For flax seed

Table 1
Determination of fatty acid contents in the NIR and MIR ranges by PLSR analysis to flax seed flours and flax seeds.

Flax seed flours														
Region	NIR							MIR						
	Cross-validation					External validation		Cross-validation					External validation	
Fatty acids	Math	LVs[a]	R^2	SEC[b]	SECV[c]	R^2	SEP[d]	Math	LVs	R^2	SEC	SECV	R^2	SEP
Linoleic	MSC + N	4	0.98	0.32	0.37	0.98	1.18	MSC + S + N	3	0.91	0.84	0.95	0.88	0.76
Linolenic	MSC + N	2	0.99	0.59	0.64	0.99	1.21	MSC + S + N	4	0.99	0.77	1.26	0.97	2.28
Linoleic	MSC + SFD	3	0.92	0.68	0.76	0.90	1.61	MSC + SFD	4	0.93	0.63	1.01	0.82	3.61
Linolenic	MSC + SFD	4	0.91	0.22	0.26	0.86	0.63	MSC + SFD	2	0.27	1.28	1.58	0.25	3.00

Abbreviations: Math: mathematical treatment; MSC, multiplicative scatter correction; N, normalization; S, smoothing; SFD, smoothing plus first derivative.
[a] Latent variables.
[b] Standard error of calibration.
[c] Standard error of cross-validation.
[d] Relative error of prediction.

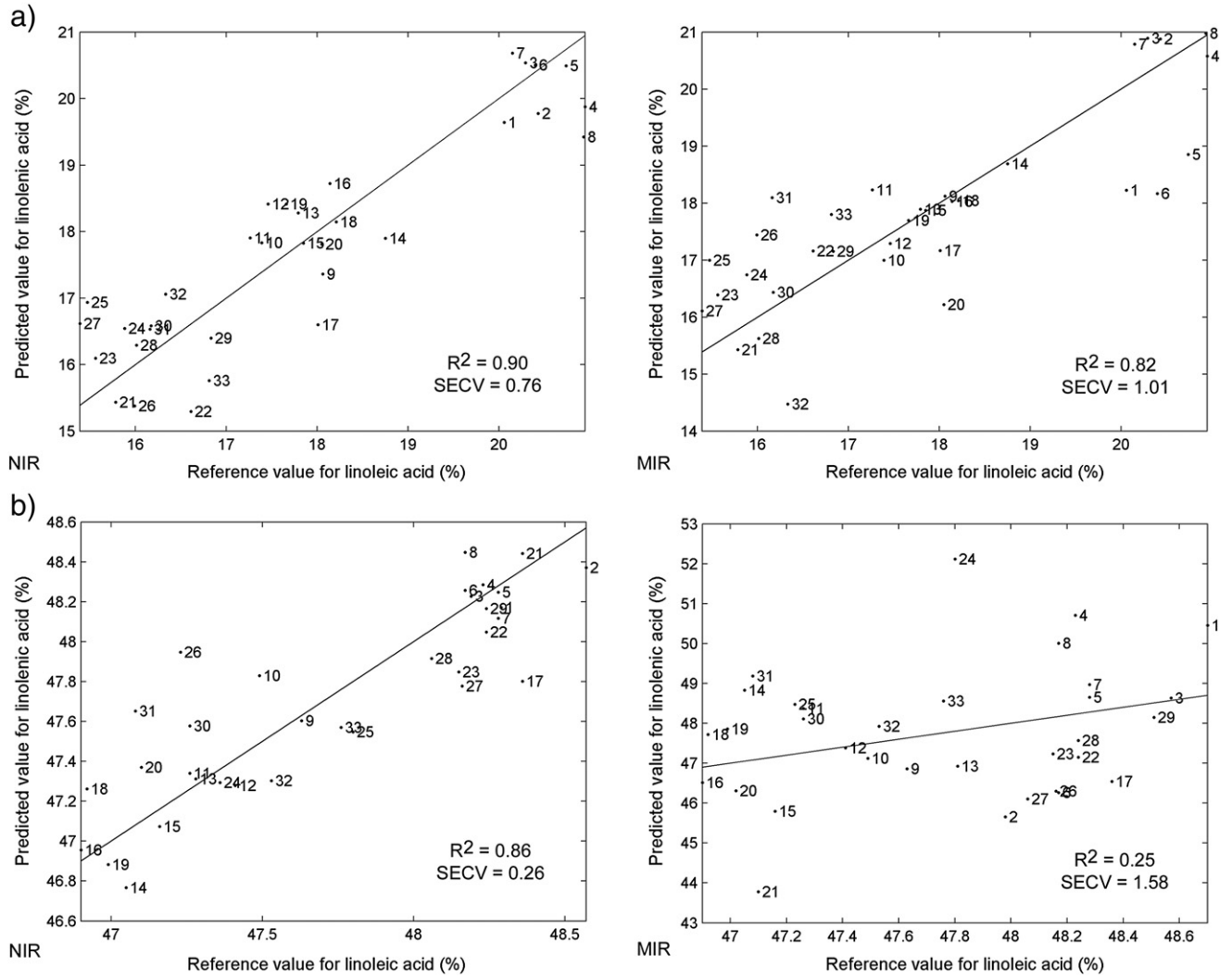

Fig. 4. Scatter plot showing the relationship between the reference value, as determined by gas chromatography (GC), and the predicted value for linoleic acid (a) and alpha-linolenic acid (b) contents by near and mid-infrared spectroscopy for flax seeds.

flour samples, the scatter plot showing the relationship between the reference value and predicted value for LA and ALA was very similar to the scatter plot presented for the flax seeds.

When absorbing radiation energy, molecules are elevated into an excited state where they vibrate to create a spectrum in the infrared region. The shape of the spectrum of any material depends on these vibration characteristics. In the NIR region, there are overtones and combination bands that involve C–H, O–H and N–H chemical bonds (Osborne, 2000), while analysis in the MIR region provides information arising from fundamental molecular vibrational frequencies (Reid, Woodcock, O'Donnell, Kelly, & Downey, 2005). NIR and MIR spectral regions have been increasingly used for qualitative and quantitative analyses of material's compositions in several fields, including the food industry (Sundaram, Kandala, & Butts, 2009). Figs. 5 and 6 present the raw and derivative spectra in the NIR and MIR regions, respectively, in which the spectral regions that contributed the most to the development of the calibration models for the LA and ALA contents were identified. Among signals for the NIR region, bands between 6950 and 7500 cm^{-1}, and between 6100 and 5800 cm^{-1}, characteristics of the first overtone and combination bands attributed to the CH_2 and CH_3 functional groups, were identified. Peaks between 5200 and 5300 cm^{-1}, associated with second overtone of C═O and O–H stretching, were also identified. For the MIR region, bands between 3600 and 3750 cm^{-1}, corresponding to O–H stretching, and bands between 1750 and 1900 cm^{-1}, associated with C═O, were identified as well (Cen & He, 2007; Lazzari & Chiantore, 1999; Pavia, Lampman, & Kriz, 2010; Silverstein, Webster, & Kiemle, 2006; Xiabo, Jiewen, Povey, Holmes, & Hanpin, 2010).

Table 2 shows the LA and ALA content, determined by the standard GC-FID analytical method, along with NIR and MIR-predicted LA and ALA contents in flax seeds and flax seed flours. The multivariate calibration models developed in this work for LA and ALA contents present better predictive capacity when compared to calibration models previously reported for the prediction of FA composition in sunflower seeds (Pérez-Vich, Velasco, & Fernández-Martínez, 1998), rapeseed (Kim, Park, Choung, & Jang, 2007) and soybean seeds (Patil et al., 2010). For the LA content, the best multivariate calibration model developed in this work for flax seeds presented lower SECV value (0.76), when compared to the SECV value previously reported for models developed for sunflower seeds (7.25, Pérez-Vich et al., 1998), rapeseed (1.3, Kim et al., 2007) and soybean seeds (1.05, Patil et al., 2010). Our model also presented lower SEP value (1.61) when compared to the SEP value previously reported for models developed for sunflower seeds (7.58, Pérez-Vich et al., 1998) and rapeseed (3.61, Kim et al., 2007), and similar value, when compared to the SEP value reported for soybean seeds (1.50, Patil et al., 2010). For the ALA content, the best multivariate calibration model developed in this work for flax seeds presented lower SECV value (0.26), when compared to the SECV

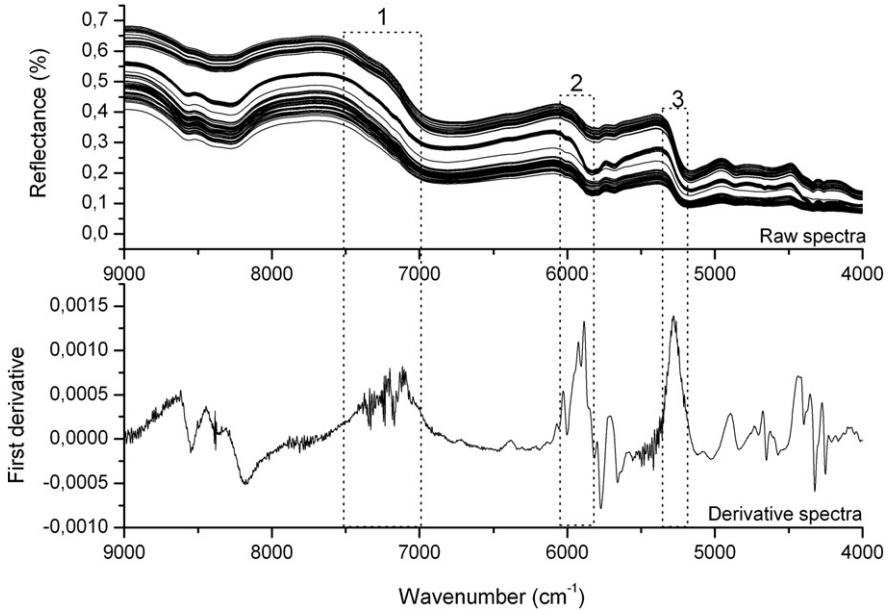

Fig. 5. Near-infrared reflectance spectra of flax seeds and flax seed flours and first derivative transformed spectra. Region 1 corresponding to bands from 6950 to 7500 cm^{-1}, and region 2 from 6100 to 5800 cm^{-1}, both are characterized for first overtone and combination bands of CH_2 and CH_3. Region 3 from 5200 to 5300 cm^{-1} associates the second overtone of C═O and O–H.

value previously reported for the model developed for rapeseed (0.79, Kim et al., 2007), and similar value compared to the value reported for soybean seeds (0.29, Patil et al., 2010). Our model also presented lower SEP value (0.63) when compared to the SEP value previously reported for models developed for soybean seeds (1.56, Kovalenko, Rippke, and Hurburgh, 2006) and rapeseed (0.76, Kim et al., 2007), though the SEP value reported for our work is higher, compared to the SEP value reported for a model described for soybean seeds (0.36, Patil et al., 2010). The multivariate calibration models developed in this work for the prediction of LA and ALA content in flax seeds and flax seeds flours present high correlation coefficients and low predictive errors, and, consequently, can be used as excellent tools to predict the LA and ALA content in flax seeds and flax seed flours.

4. Conclusions

Results from this work demonstrated that good multivariate calibration models can be developed for the prediction of ALA content in both flax seeds and flax seed flours, using spectral data from the NIR region. For LA content, good prediction models for flax seed flours can be developed using spectral data from the MIR region, while for flax seeds, the LA content can be predicted through calibration models using spectral data from the NIR region. These results also demonstrate that the use of calibration models can replace, with advantages, i.e., faster and non-destructively, with good accuracy, with minimal sample preparation and lower cost, methods traditionally used for the determination of LA and ALA content, such as gas

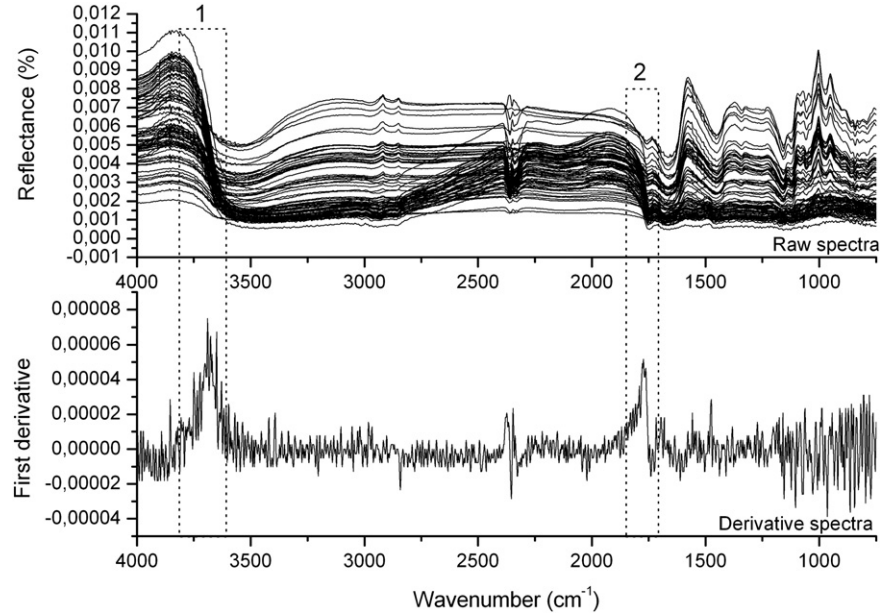

Fig. 6. Middle-infrared reflectance spectra of flax seeds and flax seed flours and first derivative transformed spectra. Region 1 corresponding to bands from 3600 to 3750 cm^{-1} associates to stretching CH_3 and O–H. Region 2, bands from 1750 to 1900 cm^{-1} are due to bands from C═O, both are characteristics from carboxylic acids.

Table 2
Fatty acid contents of flax seeds and flax seed flours measured by the standard GC-FID and infrared spectroscopy (NIR and MIR) analytical methods for the external validation.

Samples	Linoleic acid			Linolenic acid		
	GC[a]	NIR	MIR	GC[a]	NIR	MIR
Flax seed flours						
1	18.15	18.05	18.01	54.10	53.98	53.50
2	18.24	18.30	17.87	55.62	54.53	51.83
3	23.12	23.18	23.17	44.18	43.36	43.97
4	22.32	23.19	22.47	44.31	43.73	45.09
5	19.72	19.62	19.88	42.81	43.49	43.70
6	19.82	19.51	19.83	43.42	43.57	42.80
Flax seeds						
1	20.17	20.57	21.04	48.7	48.52	46.13
2	20.14	20.35	19.56	47.98	48.14	46.84
3	17.49	17.78	18.27	47.81	47.66	47.34
4	18.19	17.88	18.64	47.02	47.36	44.06
5	15.9	16.16	16.51	48.51	47.82	48.48

[a] Standard analytical method.

chromatography, especially in flax seeds and flax seed flours. Moreover, the multivariate calibration models reported in this work are expected to enable the prediction of LA and ALA content with enough accuracy for quality control purposes. These calibration models also show remote sampling capability, thus providing real-time information in a process stream.

Screening for a large number of samples by conventional gas chromatography (GC-FID) method is a major constraint in breeding programs aimed at improving oil quality through altered fatty acid composition in seeds. The calibration models presented in this work can be immediately applied for the evaluation of breeding material in ongoing breeding programs aimed at further enhancing the ALA content in flax seeds, significantly improving the efficacy of the breeding programs.

Acknowledgments

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